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14. ABSTRACT
Epidermal growth factor (EGF) receptor tyrosine kinases (erbB family), EGFR (erbB1) and HER2, are highly expressed in breast cancer and are associated with poor prognosis. A number of EGFR and/or HER2-targeted agents are being investigated for breast cancer treatment. Brk (Breast Tumor Kinase) is a nonreceptor tyrosine kinase that has been shown to enhance the mitogenic signaling of EGF, induce phosphorylation of erbB 3 and interact with AKT. In this study, we aim to investigate whether Brk can promote cells to become refractory to EGFR-targeted drugs. PI-3 kinase/AKT pathway mediates EGF-induced cell growth and survival and is involved in cellular resistance to anti-cancer drugs. Because the PI3K/AKT pathway is regulated by multiple activators, downregulation of the EGFR alone may not lead to its inhibition. We will investigate whether Brk promotes growth and survival as well as PI3K/AKT activity in cells treated with EGFR-targeted agents.

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Introduction

Epidermal growth factor (EGF) receptor tyrosine kinases (erbB family), EGFR (erbB1) and HER2, are highly expressed in breast cancer and are associated with poor prognosis. A number of EGFR and/or HER2-targeted agents are being investigated for breast cancer treatment. However, the redundancy of signaling pathways which promote cell growth and prevent apoptosis can cause cells to become insensitive to these drugs. Brk (Breast Tumor Kinase) is a nonreceptor tyrosine kinase that has been shown to enhance the mitogenic signaling of EGF, induce phosphorylation of erbB 3 and interact with AKT (refs 1-4). In this study, we aim to investigate whether Brk can promote cells to become refractory to EGFR-targeted drugs. PI-3 kinase/AKT pathway mediates EGF-induced cell growth and survival and is involved in cellular resistance to anti-cancer drugs. Because the PI3K/AKT pathway is regulated by multiple activators, downregulation of the EGFR alone may not lead to its inhibition. We will investigate whether Brk promotes growth and survival as well as PI3K/AKT activity in cells treated with EGFR-targeted agents.

We propose to undertake four different tasks, as shown in the Statement of Work. The work that has been accomplished is described in Key Research Accomplishment. We have had some difficulty in two main tasks, first, the establishment of the method to suppress Brk expression in breast cancer cells utilizing RNA interference approach. Second, the establishment of breast cancer cell lines with Brk knocked down which prevent us from investigating the effect of Brk suppression on cancer development in a mouse model. The problems and the resolution are described in the Key Research Accomplishment. Several strategies have been taken for trouble-shooting and we have accomplished a large part of the proposal. Furthermore, we have generated important data that lead to broader understanding of the function of Brk. The manuscript on this study is in preparation.

Statement of Work

Task 1. To determine the role of Brk on the efficacy of EGFR inhibitors to antagonize cell growth and survival:

1.1). Human breast cancer cell lines will be selected by a RT-PCR screen for cells which overexpress both Brk and EGFR.

1.2) shRNA targeting to 'knock down' Brk gene expression will be designed and transfected into selected cell lines from 1.1. Cells will be tested for the levels of mRNA and protein expression of Brk. Subsequently, two Brk-knockdown clonal cell lines will be established.

1.3) The clonal cell lines, the Brk-knockdown and parental cells, will be treated with different EGFR inhibitors including AG1478, ZD1839 and GW572016. The duration of the treatment will be approximately 2 weeks. The rate of cell proliferation and apoptosis will be assessed during the course of the treatment.

Task 2. To determine whether Brk overexpression induces cells to become refractory to the EGFR inhibitors.

2.1). Human breast cancer cell lines will be selected by a RT-PCR screen for cells which contain high levels of endogenous EGFR and low or undetectable levels of endogenous Brk.

2.2). Brk will be transfected into selected cell lines from 2.1 and tested for the level of Brk protein expression. Two clonal cell lines overexpressing Brk will be established.

2.3). The clonal cell lines overexpressing Brk and parental cells will be treated with EGFR inhibitors AG1478, ZD1839, and GW 572016. The duration of the treatment will be approximately 2 weeks. The rate of cell proliferation and apoptosis will be assessed during the course of the treatment.

Task 3. To test whether Brk upregulates PI3K and AKT activity in the cells exposed to EGFR inhibitors.

The Brk knockdown, overexpressed, and parental cells, established in Task 1 and 2, will be treated with AG1478, ZD1839, or GW 572016. Subsequently, cells will be analyzed for the PI3K and AKT activity at various time-points (day1-14). The analysis will be achieved by a standard *in vitro* PI-3 kinase assay and measurement of the levels of phosphorylated AKT by immunoblotting.

Task 4. To determine the role of Brk on the efficacy of EGFR inhibitors to antagonize cell growth and survival in an *in vivo* setting.

4.1). The Brk-knockdown clonal cells, established in 1.2, and parental cells will be injected subcutaneously into female BALBc $^{nu/nu}$ mice and they will be monitored for tumor establishment. Two cell lines will be utilized. 12 mice will be utilized for each experimental condition. The experimental conditions will be as follow: a) mice injected with Brk-knockdown cells and treated with drug, b) mice injected with Brk-knockdown cells but not treated with drug, c) mice injected with parental cells and treated with drug, d) mice injected with parental cells but not treated with drug. Forty-eight mice will be utilized in the experiment. Two cell lines will be injected into the animals, therefore, total of 96 mice will be utilized in the entire experiment.

4.2) Following tumor establishment, mice will be administered ZD1839 by oral gavage. After the course of drug treatment (3-4 weeks), mice will be sacrificed. Tumor size will be measured, and tumor growth rate will be assessed and compared between drug-treated and untreated cells.

Key Research Accomplishment

Task 1. To determine the role of Brk on the efficacy of EGFR inhibitors to antagonize cell growth and survival

1.1) Selection of human breast cancer cells.

Fifteen breast cancer cell lines were screen for cells that express high levels of both Brk and EGFR. These cell lines will be utilized for the investigation of the effect of Brk on cell response to EGFR inhibitors. The levels of Brk and EGFR expression in the cell lines were detected by western blot analysis. Three breast cancer cell lines were selected to be utilized in the study, the selection was based on their high levels of the expression of Brk and EGFR. These cell lines include T47D, SKBr3, and MDA-MB-231 (Fig.1).

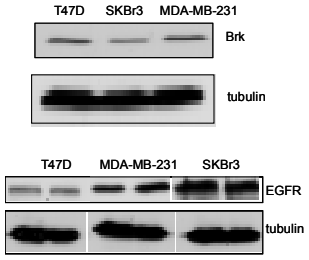

Fig. 1. Cell lines T47D, SkBr3 and MDA-MB-231 were harvested, cells were lysed and equal amount of the cell lysate were subjected to protein fractionation by SDS-PAGE. Brk, EGFR, and tubulin were detected by western blot analysis, utilizing anti-Brk, anti-EGFR and anti-tubulin antibodies.

1.2). Designing of RNA interference (RNAi) targeting to knockdown Brk

We designed 3 different shRNA sequences and constructed them into the pSUPER vector to generate pSUPER shRNA (pSUPER shRNA-Brk) targeting Brk at 3 different target sites. Additionally, a scramble RNA sequence (pSUPER shRNA-sc) were designed. The efficiency of Brk knockdown was tested by transfection of pSUPER shRNA-Brk or pSUPER shRNA-sc into the three selected breast cancer cells, T47D, SkBr3, and MDA-MB-231. Three days after the transfection, the cells were harvested and the cell lysate were subjected to analysis of Brk protein level by western blot analysis with anti-Brk antibody. All three different shRNA-Brk constructs were unable to sufficiently suppress the Brk expression (data was presented in the first annual report). We redesigned the RNA sequence by utilizing microRNA (miRNA) instead of shRNA. This method has been shown to yield better stability of the RNA interference. Three sequences of miRNA targeting to knockdown Brk and one control sequence were constructed in pSM155 vector. Subsequently, the effect of these miRNAs on Brk expression was tested. Less than 20% suppression of Brk was observed in the cells transfected with these miRNA-Brk (data not shown). Since 20% suppression of Brk is insufficient for our purpose, we were unable to use these miRNA. To circumvent the problem, we designed the synthesized siRNA. siRNA targeting Brk (siRNA-Brk/kd) and scramble RNA

(siRNA-sc) were purchased from Dharmacon Inc. The siRNA was transfected into the cell lines T47D, SkBr3 and MDA-MB 231. A significant reduction of Brk expression was observed in T47D (approximately 60% of control), and detectable level of reduction was yielded in SkBr3 and MDA-MB-231 (40-50% of control) (Fig. 2).

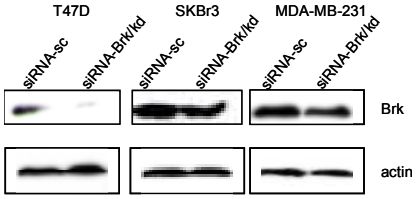

Fig. 2. Cell lines T47D, SkBr3 and MDA-MB-231 were transfected with siRNA-Brk/kd or siRNA-sc. Two days after transfection, cells were harvested, cells were lysed and equal amount of the cell lysate were subjected to protein fractionation by SDS-PAGE. Brk was detected by western blot analysis, utilizing anti-Brk, antibody. A fraction of the cell lysate from each sample was taken and loaded on a separate gel to probe for actin utilizing anti-actin antibody. This presented data represents the results of three independent experiments.

1.3) Determination of the role of Brk on cell proliferation

To determine the effect of brk knockdown on cell proliferation, we transfected the siRNA targeting Brk (siRNA-Brk/kd) or control (siRNA-sc) in three breast cancer cell lines, and assessed the number of viable cells by performing MTT assay. Brk knockdown induces significant decrease of cell proliferation in various breast cancer cell lines, as shown in Fig. 3. These data indicate that Brk plays an important role in the regulation of cell proliferation, which is consistent with previous report that Brk promotes cell proliferation (1).

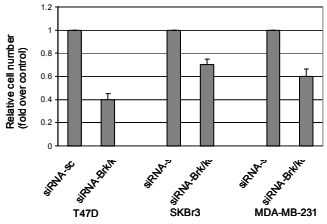

Fig. 3. Cells were transfected with siRNA-Brk or siRNA-sc. Three days after transfection cells were subjected to standard MTT assay and the MTT activity was measured by microplate reader, to measure the relative number of viable cells. Graph represents relative number of viable cells. Data represent mean values of three independent experiments.

Task 2 To determine whether Brk induces cells to become refractory to the EGFR inhibitors

Since knockdown of Brk induces a significant decreased proliferation, it suggests that Brk has an important role in the regulation of cell proliferation. We further determined whether Brk could induce proliferation of cells treated with EGFR-targeted cancer drugs, Traceva, an EGFR inhibitor, and GW2974, a HER1/HER2 dual inhibitor. MDA-MB-231 cells were transfected with siRNA targeting Brk or scramble RNA . Subsequently, the transfected cells were treated with Traceva, GW2974 or vehicle for 2.5 days. Cell proliferation rate were measured by MTT assay. GW2974 inhibits cell proliferation with the IC_{50} of 50 uM in control cells and 80uM in Brk transfected cells (Fig. 4). This data indicates that Brk interferes with the mechanism of inhibition of cell proliferation by GW2974. Traceva treatment has no inhibitory effect on proliferation of either control or Brk-knockdown cells (data not shown). A similar set of experiment was carried out in cells that are transfected with siRNA targeting Brk or scramble control. Since Brk interferes with the inhibitory effect of GW2974, we predicted that suppression of Brk expression will sensitize the drug action. To our surprise, suppression of Brk has no significant effect on cellular sensitivity to GW2874. Further investigation will address whether there are alternative pathways that allow cells to circumvent the disruption of Brk signaling.

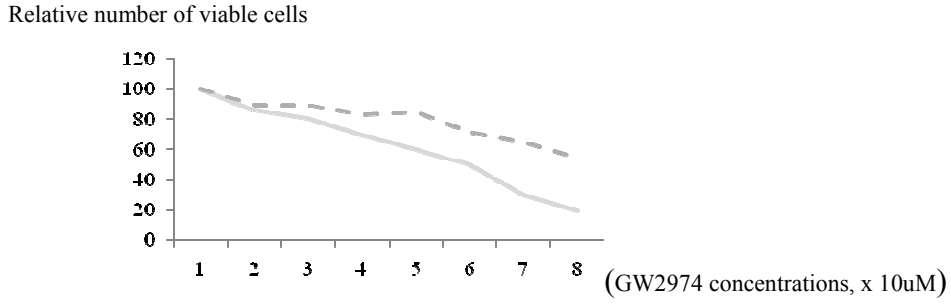

Fig. 4. pCGNBrk or pCGN vector was transfected into MDA- MB-231cells and cells were treated with GW2974 (20 ,40, 60, 80, 100 uM) or control vehicle (DMSO). Cells were maintained for 2.5 days followed by MTT assay. The absorbance was read by microplate reader. Graph represents relative number of viable cells. Data represent mean values of three independent experiments. Solid line = control cells; broken line = Brk expressing cells.

Task 3 Determination of the role of Brk in the regulation of Akt activity

Akt regulates cell growth and survival and induces cell resistance to cancer drugs. Since Brk signaling pathway is associated with Akt, we will examine whether Brk can induce Akt activation and whether the mechanism is dependent on EGFR (2,3,4). We have documented in the previous progress report that Brk promoted the activation of Akt as judged by the increase of phosphorylation of the regulatory site of Akt, serine-473. Furthermore, we found that, in MDA-MB-231 cells, this increase of AKT phosphorylation appears to be EGF independent, since there was a detectable increase in cells expressing Brk compared to control in the absence of EGF stimulation. In T47D cells, however, the increase of AKT phosphorylation is EGF dependent. Since the activation of Akt in T47D cells appear to depend on EGF, we further define the pathway, as to whether it is dependent on HER1 and HER2.

T47D cells were transfected with pCGNBrk or pCGN vector. One day after transfection, cells were treated with GW2974 and incubated for 16 hours. Cells were harvested and the cell lysate were subjected to protein fractionation by SDS-PAGE and AKT was detected by immunoblot analysis using an antibody specific to AKT. The phosphorylated form of Akt-serine-473 was detected by the antibody specific to phosphor serine 473 of Akt. The result showed a detectable increase of AKT phosphorylation in cells transfected with Brk compared to control and this increase is abolished when treated with GW2974 (fig. 5). This result suggests that the regulation of Akt by Brk is dependent on HER1 and HER2, in T47D cells.

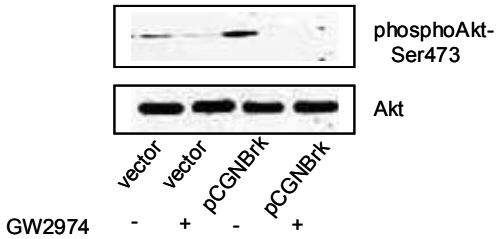

Fig. 5. T47D cells were transfected with pCGNBrk or pCGN vector. One day after transfection, cells were treated with GW2974 and incubated for 16 hours. Cells were harvested and equal amount of the cell lysate were subjected to protein fractionation by SDS-PAGE and western blot analysis. The phosphorylated form of Akt-serine-473 was detected by the antibody specific to phospho-Akt-Ser-473. A fraction of the cell lysate from each sample was taken and loaded on a separate gel to probe with anti- AKT antibody. This data represents the results of three independent experiments.

Brk regulates cell cycle

Based on our finding that Brk promotes cell proliferation in the presence of the HER1/HER2 inhibitor, raised the question whether Brk altered the regulation of cell cycle. Furthermore, our result shows that Brk can activate Akt in the absence of serum in MDA-MB-231, which suggests that Akt may be a key component in inducing cell resistance to the drug. Akt is a negative regulator of the Forkhead Box O (FoxO) family of transcription factors. FoxO proteins induce expression of negative regulators of G1/S cell cycle, including the key cell cycle inhibitor p27. Growth factor deprivation induces FoxO nuclear translocation and subsequent gene transcription, whereas growth factor stimulation induces the nuclear export of FoxO proteins, allowing quiescent cells to reenter G1 phase of cell cycle. Akt mediates sequestration of FoxO in the cytoplasm, thus inhibits transcription of cell cycle inhibitors. We first examined whether Brk inhibited the nuclear localization of FoxO proteins, focusing the attention on FoxO3a, the major FoxO family protein in MDA-MB-231 cells. MDA-MB-231 cells were transfected with pCGN-HA-Brk plasmid or vector, and cells were serum starved for 40 hours before subjected to nuclear fractionation. The nuclear fraction was subjected to SDS-PAGE and western blot analysis. As shown, the level of the nuclear-localized FoxO3a is significantly decreased in cells overexpressing Brk (Fig. 6, a and b). This data indicates that Brk inhibits the nuclear localization of FoxO3a. Next, we examined whether Brk influence the expression of p27. MDA-MB-231 cells were transfected with Brk, followed by 40-hour serum starvation and the levels of p27 in the whole cells lysate were assessed by western blot analysis. Expression of Brk induces a significant decrease of p27 expression (fig. 6, c and d). Similarly, suppression of endogenous Brk with siRNA causes the increase of p27 level (fig. 6, e and f). Together, these data show that Brk inhibits the nuclear localization of FoxO3a and

downregulates p27 expression. Since Brk activates Akt, it is likely that the regulation of FoxO3a is mediated by Akt. In conclusion, we have demonstrated that Brk regulates subcellular localization of FoxO3a. By inhibiting FoxO3a to accumulate in the nucleus, Brk inhibits the expression of p27, a FoxO gene target. Since p27 is a crucial cell cycle inhibitor which expresses upon growth factor deprivation, this mechanism may underlie the alteration of cellular sensitivity to GW2974 in Brk expressing cells.

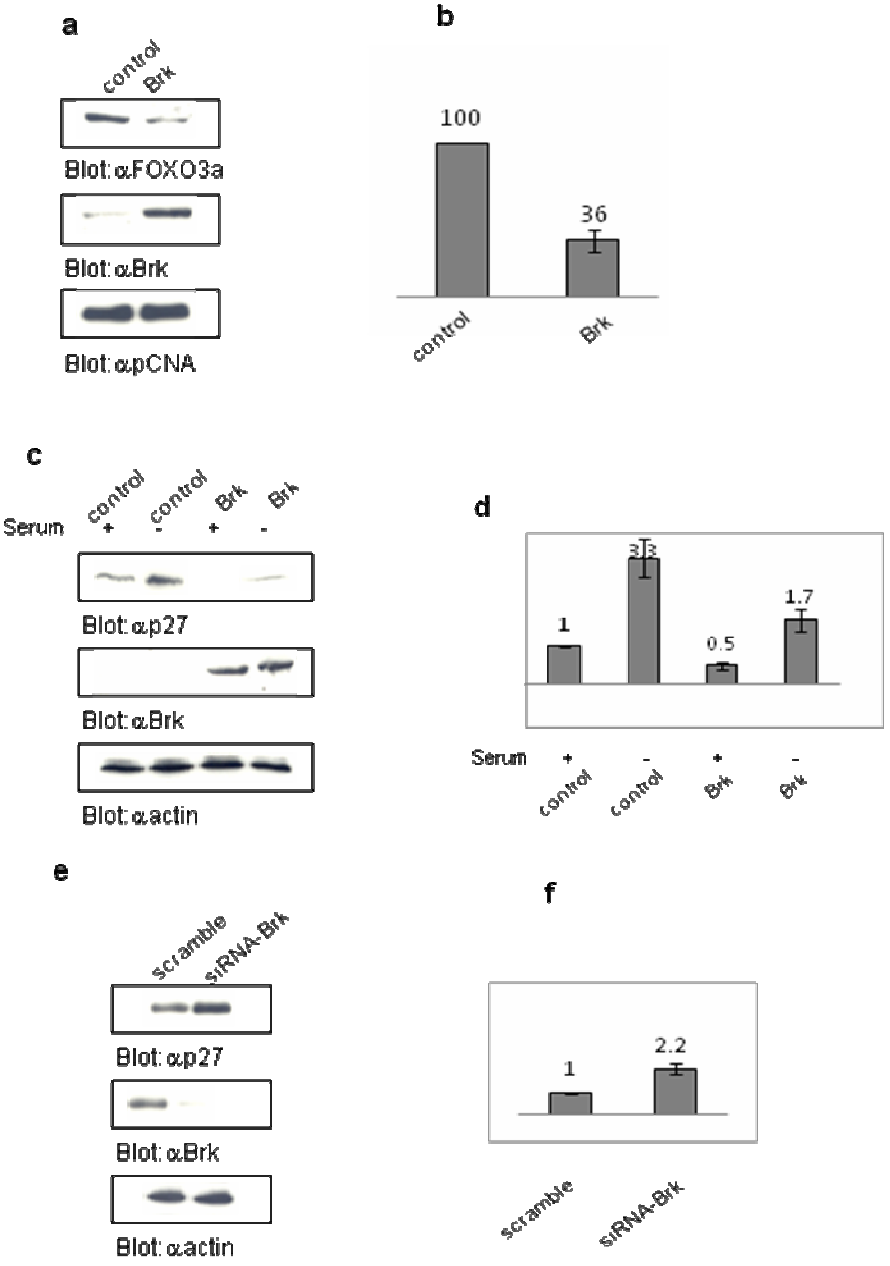

Fig. 6 a and b, pCGN-HA-Brk or vector was transfected into MDA-MB-231 cells, and cells were serum starved for 40 hours. The cell lysates were subjected to nuclear fractionation and the nuclear localized FoxO3a was fractionated by SDS-PAGE and analyzed by western blot analysis, utilizing anti-FoxO3a antibody. Brk and pCNA (loading control) were detected by the specified antibodies. **c and d,** pCGN-HA-Brk or vector was transfected into MDA-MB-231 cells, and cells were either maintained in the culture media containing 10% fetal bovine serum (FBS) or serum starved for 40 hours prior to harvest. The whole cell lysates were subjected to SDS-PAGE and western blot analysis, utilizing anti-FoxO3a, Brk and actin (loading control) antibodies. **e and f,** MDA-MB-231 cells were transfected with siRNA-Brk or scramble RNA and the cells were serum starved for 40 hours prior to harvest. The cell lysates were subjected to SDS-PAGE and western blotting with anti-p27, Brk, and actin antibodies. Graphs represent values of nuclear-fraction FoxO3a (b) and p27 expression (d and f), relative to control, data are mean values of three independent experiments.

Summary

The finding from this study includes
1. Brk induces cell proliferation.
2. Brk induces the decrease of cellular sensitivity to GW2974.
3. Brk induces the phosphorylation of Akt in an EGF-independent manner in MDA-MB-231 cells
4. Brk negatively regulates the activity of the transcription factor FoxO3a which results in down-regulation of the cell cycle inhibitor p27.

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